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Role of neuron-specific splicing regulators as modifiers of neurofibromatosis type 1

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14. ABSTRACT

We hypothesized that the tightly regulated ratio of NF1 isoforms is critical in maintaining the homeostasis of cell growth and differentiation, thereby determining the functional output of NF1 gene. A corollary is that splicing regulators may modify the function of neurofibromin through altering alternative splicing of the NF1 pre-mRNA.

We propose two specific aims to study the role of regulated alternative splicing in the function of neurofibromin. In **Aim I,** we will determine how changes in alternative splicing affects NF1 function. In **Aim II**, we will determine the biological consequence of altering the ratio of neurofibromin isoforms in cells with natural NF1 expression such as neuronal and glial cells.

To this end, we have identified the cis-acting elements located to the vicinity of exon 23a that play key roles in regulating inclusion of this exon and tested the effects of mutations of these elements in primary neurons. We also generated the gene-targeting vector to create a knock-in allele at the NF1 locus in mouse ES cells and are in the process of screening ES cell clones that have the correctly targeted allele. In addition, we successfully established the ES cell differentiation procedure.

Results of the proposed studies will not only provide important novel insights into the etiology of NF1 disease, but also shed light on how genetic variations in splicing regulators affect the progression of other diseases.

15. SUBJECT TERMS
Alternative splicing, NF1 exon 23a, splicing signals, gene-targeting

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INTRODUCTION

We hypothesize that the tightly regulated ratio of NF1 isoforms is critical in maintaining the homeostasis of cell growth and differentiation and thus, it determines the functional output of NF1 gene. Furthermore, splicing regulators may modify the function of neurofibromin through altering alternative splicing of the NF1 pre-mRNA. There are two main goals of the proposed studies. We will first investigate how alternative splicing modulates the function of neurofibromin. Particularly, we will determine how cis-acting RNA sequence elements and trans-acting splicing regulators modulate the function of neurofibromin by examining not only the splicing status of exon 23a but also measuring the Ras activity, the functional output of altered NF1 splicing. The second goal of these studies is to determine the biological consequence of altered ratio of the two neurofibromin isoforms. We will create a unique system that will allow us to readily modify the NF1 alleles. We will use this system to generate mouse ES cell lines that express the two NF1 isoforms at different ratios. Subsequently, we will use a recently developed differentiation strategy to derive neural stem cells from these ES cells to examine the cell growth and differentiation potential of these cells.

BODY

During the last funding period, we made progress in three areas towards the goals of the two specific aims. First, in the first year of this grant, we identified the point mutations at both acceptor and donor site of exon 23a, which will ensure predominant inclusion of this exon in all cell types (Aim Ia). We now have further tested these mutations using our splicing reporter in primary neurons. We showed that the point mutations resulted in near complete inclusion of exon 23a in mouse cerebellar neurons. These results gave us tremendous confidence that our knock-in strategy will lead to expected splicing results. Second, we generated the knock-in targeting vector that harbors the engineered mutations and transfected mouse ES cells. We are in the process of screening colonies to find the clones with the targeted allele (Aim IIa). Third, we successfully adopted the procedure that differentiates mouse ES cells into neurons (Aim IIb). This procedure will be used to analyze ES cells with altered NF1 alleles.

Aim I. To determine how altered alternative splicing affects NF1 function. Our preliminary data indicate that Hu proteins and TIAR are involved in the regulation of exon 23a inclusion. In **Aim Ia**, we will investigate the mechanism by which the cis-acting RNA regulatory sequence on NF1 pre-mRNA regulates inclusion of exon 23a. Multiple NF1 reporter constructs carrying mutations of key regulatory sequence elements will be generated and tested for splicing by transfecting two types of cultured cells, one that processes the NF1 pre-mRNA to predominantly include exon 23a, and the other that processes to predominantly exclude this exon. The results of these experiments will not only test the contribution of the cis-acting regulatory sequences to regulated alternative splicing, but also lay the foundation for the study in the Aim IIa. The goal is to create several mini-gene reporters that, in transfected cells, will be processed to have differing ratios of exon 23a+/exon 23a- ranging from predominantly exon 23 inclusion to predominantly exon 23 exclusion. **In Aim Ib**, we will change expression level of Hu proteins or TIAR and examine the function of neurofibromin. We will either over-express tissue culture cells that have little expression of Hu proteins or knockdown the expression of these proteins in

cells that express these proteins and then determine how alternative splicing involving exon 23a is affected and how Ras activity output is affected.

Progress

1. Contribution of splice site strength to regulated inclusion of NF1 exon 23a (Aim Ia).

In general, alternatively included exons tend to have suboptimal splicing signals to allow regulation. To understand which the relative contribution of each of the splicing signals of exon 23a, we mutated these sequences individually and in combination in the NF1 reporter constructed generated in the laboratory as shown in last year's report and Fig. 1A. We tested these mutations in a NF1 splicing reporter in transfected primary neurons (Fig. 1B).

As predicted, when the splice donor or/and acceptor was mutated to their consensus sequences, exon 23a inclusion was significantly increased in transfected neurons (Fig. 1B).

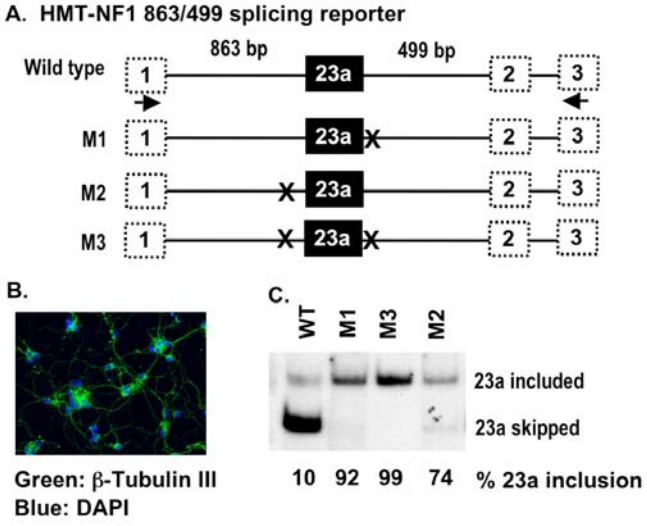

Figure 1. A. HMT-NF1 splicing reporter containing exon 23a with 863 bp upstream and 499 bp downstream intronic sequence inserted into the first intron of HMT. X indicates mutation. Primers for RT-PCR analysis of exon 23a inclusion are located in HMT exons 1 and 3. **B.** Mouse cerebellar neuron culture. **C.** Splicing analysis of wild type and mutant NF1 reporters in cerebellar neurons by RT-PCR.

2. Effect of altered levels of trans-acting factors (Hu proteins or TIAR) on the function of the NF1 gene product

To determine if the biological output of the NF1 expression will be changed as a result alternative splicing change of exon 23a, we planned to alter expression of its regulators. We started with the Hu proteins. The relative low transfection efficiency makes it very difficult to assess the effect on over-expressed Hu protein on functional output of NF1, i.e., Ras signaling, we decided to use the lentiviral tranduction system to carry out this experiment. Last year we reported that we cloned the mHuB and mHuC into a lentiviral vector, packaged the expression vectors into virus particles. Unfortunately, the expression level of Hu proteins in cells after infecetion is very low, which made the next-step analysis very difficult. We need to find other ways to do this experiment. However, since we will analyze how splicing change in NF1 affect Ras signaling in targeted ES cells, we will come back to this issue at that time.

Aim II. **To determine the biological consequence of altered ratio of neurofibromin isoforms in cells with natural NF1 expression such as neuronal and glial cells.** In **Aim IIa**, to examine the NF1 function in its natural context, we will integrate the engineered sequences determined by Aim Ia that lead to differing ratios of exon 23a+/exon 23a- into the endogenous NF1 allele using gene targeting technology. An elegant state-of-the-art strategy will be used to obtain targeted ES cell lines with a series of different NF1 alleles. In **Aim IIb**, we will analyze the growth and differentiation potential of cells harboring the modified NF1 alleles. Neural stem cells (NTC) will be derived from these ES cells and analyzed for their growth potential in

comparison t o t hat der ived f rom wi ld t ype ES cel ls us ing a r ecently des cribed E S cel l differentiation procedure.

Progress

Generate the targeting vector (Aim IIa)

We designed the strategy to create the targeting vector as outlined in year's report. We have successfully generated the targeting vector using the BAC modification system as planned. The targeting vector was given to the Transgenic Core facility in the Medical School at Case Western Reserve University to generate the targeted mouse ES cells. We got colonies from the facility and are in the process of screening for correctly targeted ES cells.

Mouse ES cell differentiation into neurons (Aim IIb)

To analyze ES cells with knock-in NF1 alleles, we need to differentiate ES cells into neural progenitors and then neurons. We have successfully adopted the procedure published by the Barde laboratory. As shown in Fig. 2, we have obtained neurons from ES cells at high efficiency. More importantly, splicing of exon 23a from the endogenously expressed NF1 follows an expected pattern, i.e., predominantly skipping in differentiated neurons. With this differentiation procedure, we are ready to proceed to analysis of the ES cells with knock-in NF1 alleles.

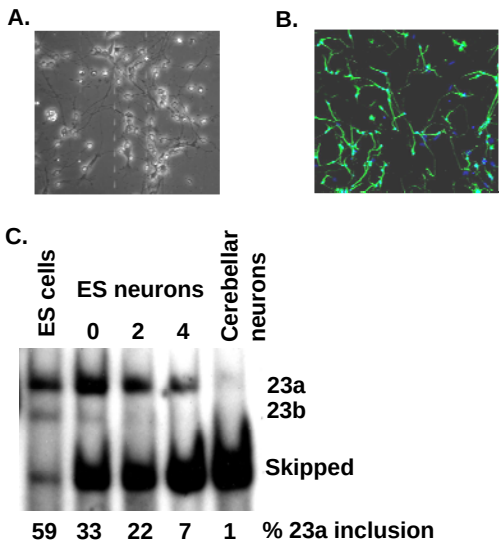

Figure 2. A. Neurons differentiated from mouse ES cells. **B.** ES neurons stained with β-tubulin III (green) and DAPI (blue). **C.** Splicing of endogenous NF1 in ES cells, differentiated neurons and cerebellar neurons analyzed by RT-PCR.

KEY RESEARCH ACCOMPLISHMENTS

-Tested point mutations that will ensure predominant inclusion of the NF1 exon 23a in primary neurons.

-Generated the NF1 knock-in targeting vector and are on our way to obtain targeted ES cells.

-Successfully differentiated mouse ES cells into a specific lineage of CNS neurons.

REPORTABLE OUTCOMES

Publication:
Hinman MN and Lou H (2008). Diverse molecular functions of Hu proteins. **Cell. Mol. Life Sci.** Jun 26. [Epub ahead of print]. Invited review (peer-reviewed).

Abstracts:
Melissa N. Hinman, Hui Zhu, Kavita Praveen, and Hua Lou. "Mammalian Hu protein family members have non-redundant functions as splicing suppressors". **RNA Society Meeting**, Berlin, Germany, July 28-August 3, 2008. Poster presentation.

Melissa N. Hinman, Hui Zhu, Kavita Praveen, and Hua Lou. "Mammalian Hu protein family members have non-redundant functions as splicing suppressors". **Rustbelt RNA Meeting**, Deer Creek State Park and Resort, Mt. Sterling, OH, October 17-18, 2008. Poster presentation.

CONCLUSION

The results presented in this report indicate that we are well on our way to reach the goals of the proposed studies for this grant. The reagents and tools generated from the past funding period will be very useful to carry out the next-step experiments towards the goals of the proposed studies. The most significant finding was that we were able to change the alternative exon 23a into a constitutive exon that can be almost completely included in primary neurons where this exon is skipped by manipulating the splicing signals surrounding this exon. The implication for the NF1 disease is that if a mutation falls within these splicing signals, it may impact how this exon is spliced. Because this exon encodes part of the GAP-related domain, the potential biological outcome will be important.

REFERENCES

None

APPENDICES

None